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(54) Title: METHOD FOR SCREENING FOR INHIBITORS AND ACTIVATORS OF TYPE III SECRETION MACHINERY IN GRAM–NEGATIVE BACTERIA

(57) Abstract

This invention relates to mutant strains of gram–negative bacteria that constitutively secrete proteins via the type III secretion machinery and to methods of identifying molecules that are able to activate or inhibit secretion in wild–type strains of gram–negative bacteria by exposing gram–negative bacterial cells to a sample molecule, wherein said bacterial cells contain a reporter gene transcriptionally fused to a promoter of a gene activated or regulated by the type III secretion machinery and detecting the presence or activity of the product of the reporter gene.

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METHOD FOR SCREENING FOR INHIBITORS AND ACTIVATORS OF TYPE III SECRETION MACHINERY IN GRAM-NEGATIVE BACTERIA

This invention relates to the field of biochemical assays involving regulated expression of reporter genes, and to mutant strains of bacteria useful in biochemical assays. More particularly, it relates to methods of screening for molecules capable of affecting expression and/or activity of type III secretion machinery in gram-negative bacteria.

Type III secretion machinery is present in numerous gram-negative bacteria (including members of the species *Shigella, Salmonella, Yersinia, Escherichia, Pseudomonas, Xanthomonas, Ralstonia,* and *Erwinia)* that are pathogenic for man, animals, and plants. For example, the Sec-independent type III secretion pathway is involved in secretion of *Yersinia* anti-host proteins. In *Salmonella* and *Shigella* species, it is involved in the process of entry into epithelial cells. It is also implicated in EPEC signal transducing proteins, *Pseudomonas aeruginosa* toxins, and virulence factors of many plant pathogens, as well as in flagellum assembly of bacteria such as *S. typhimurium* and *Bacillus subtilis*.

Features of this secretion pathway can include activation of secretion by contact of the bacterium with host cells (Ménard *et al.*, 1994, "The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD.", *EMBO J.*, **13**:5293-5302; Watarai *et al.*, 1995, "Contact of *Shigella* with host cells triggers release of Ipa invasins and is an essential function of invasiveness.", *EMBO J.*, **14**:2461-2470; Zierler and Galan, 1995, "Contact with cultures epithelial cells stimulates secretion of *Salmonella typhimurium* invasion proteins InvJ.", *Infect. Immun.*, **63**:4024-4028); that some of the secreted proteins are delivered into the cytoplasm of host cells (Rosqvist *et al.*, 1994, "Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells.", *EMBO J.*, **13**:964-972; Sory and Cornelis, 1994, "Translocation of an hybrid

YopE-adenylate-cyclase from *Yersinia enterocolitica* into HeLa cells.", *Mol. Microbiol.*, **14**:583-594; Wood *et al.*, 1996, "SopE, a secreted protein of *Salmonella dublin*, is translocated into the target eukaryotic cell via a *sip*-dependent mechanism and promotes bacterial entry.", *Mol. Microbiol.*, **22**:327-338; Collazo and Galan, 1997, "The invasion-associated type III system of *Salmonella typhimurium* directe the translocation of Sip proteins into the host cell.", *Mol. Microbiol*, **24**:747-756); and that transcription of genes encoding secreted proteins is controlled by secretion of regulatory proteins (Hughes *et al.*, 1993, "Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator.", *Science*, **262**:1277-1280; Pettersson *et al.*, 1996, "Modulation of virulence factor expression by pathogen target cell contact.", *Science*, **273**:1231-1233).

Based on the observations that (1) the secretion machinery is involved in secretion of factors which are active against the host, and (2) secretion mutants are avirulent, the type III secretion machinery provides an attractive target for the screening of molecules that would prevent or inhibit gram-negative bacteria from secreting their virulence factors. However, the search for molecules capable of inhibiting the secretion mechanism has previously required two conditions to be present. First, the type III secretion machinery must be active. And second, the product of the secretion activity, *i.e.*, the secreted proteins, must be measurable. Unfortunately, the secretion machinery is, at best, only weakly active when bacteria are grown in standard laboratory media, making the search for inhibitor molecules difficult or impossible. In addition, there is no way to easily measure the presence of a protein secreted in the culture medium by the type III secretion machinery. These proteins do not have an easily assayable enzymatic activity and their secretion must be evaluated using ELISA, which is time consuming and expensive.

This invention provides mutant strains of gram-negative bacteria that constitutively secrete proteins via the type III secretion machinery.

This invention also provides methods of identifying molecules that are able to activate or inhibit secretion in wild-type strains of gram-negative bacteria. These methods comprise the steps of :

a) exposing gram-negative bacterial cells to a sample molecule, wherein said bacterial cells contain a reporter gene transcriptionally fused to a promoter of a gene activated or regulated by the type III secretion machinery; and

b) detecting the presence or activity of the product of the reporter gene.

To practice the methods of this invention, genes under transcriptional control of the type III secretion machinery are identified. Transcriptional fusions between the promoters of these genes and a reporter gene, such as the *lacZ* reporter gene, are constructed and introduced into wild-type gram-negative bacteria and mutants of these bacteria that constitutively secrete proteins via the type III secretion machinery or are deficient for secretion via the type III secretion machinery. The presence (or activity) of the reporter gene product is evaluated under conditions leading to active secretion to demonstrate that the transcriptional activity of these promoters can be used as an indicator of the secretion activity of the type III secretion machinery.

Using *Shigella* as a model system for the screening of inhibitors of type III secretion, five genes under transcriptional control of the type III secretion machinery have been identified and the promoters of these genes have been used to create transcriptional fusions with the reporter gene, *lacZ*. β-galactosidase activity can be induced in recombinant *Shigella* cells harboring these transcriptional fusion constructs under conditions known to lead to active secretion.

Any gram-negative bacteria containing type III secretion machinery may be used in the methods of this invention. Suitable bacteria include members of the species *Shigella, Salmonella, Yersinia, Escherichia, Pseudomonas, Xanthomonas, Ralstonia,* and *Erwinia*. Similarly, any suitable reporter gene may be used to create a transcriptional fusion construct for use in the methods of this invention. Some suitable reporter genes are, for example, *lacZ*, *phoA*, *luxAB*, and *gfp*. In a preferred method of this invention, the reporter gene is the *lacZ* gene.

In one method of the invention, the promoter is from a gene selected from the group consisting of *virA* and the *ipaH* family of genes, particularly, *ipaH9.8*, *ipaH7.8*, *ipaH4.5*, and *ipaH1.4*. Promoters such as *ipgD*, *icsB*, *ipaA*, and *mxiD* are not regulated by the secretion machinery and thus may be used in the methods of this invention as internal controls. Other suitable promoters for use in the methods of this invention may be easily identified following the teachings detailed in this specification.

In a preferred method according to this invention, candidate inhibitor molecules are screened against three strains of bacteria which contain a reporter gene transcriptionally fused to a promoter of a gene regulated by the activity of the type III secretion machinery : a strain in which secretion is regulated, a strain which has a phenotype of constitutive secretion, and a strain which is deficient for secretion.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts secretion of proteins by various *Shigella* strains. Cultures of M90T (wild type), BS176 (the virulence plasmid-cured strain), and the *ipaD* (SF622), *ΔipaBCDA* (SF635), and *ipaD mxiD* (SF634) mutants were used to prepare either whole culture extracts, by adding Laemmli sample buffer directly to the cultures, or bacterial pellets and culture supernatants, by centrifugation of the cultures. Proteins present in culture supernatants were concentrated 10 times by TCA precipitation. Samples were separated by SDS-PAGE and analyzed by either Coomassie blue staining or immunoblotting using an antiserum raised against aggregated recovered from the medium of the *ΔipaBCDA* mutant. Numbers indicate the position and the size (in kDa) of standard proteins and arrows indicate the position of the 60- and 46-kDa proteins.

Figure 2 depicts the structure of plasmids carrying *virA* and *ipaH9.8*. A schematic genetic map of a portion of the virulence plasmid pWR100 is shown in the center, along with the position of some relevant restriction sites. Symbols used for restriction sites are : B, *Bsp*EI; C, *Hinc*II; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I; P, *Hpa*I; S, *Sau*3AI; T, *Stu*I; V, *Bbv*I; X, *Xba*I. The DNA corresponding to *virA* and *ipaH9.8* is shown by shaded bars and the *lacZ* gene by a solid bar. Arrows indicate the

orientation of transcription of the genes. Restriction sites of the virulence plasmid that were used for cloning are indicated in brackets.

Figure 3 depicts transcription of the *virA-lacZ* fusion upon addition of Congo red to the growth medium. Congo red (100 µg/ml) was added to the growth medium during the exponential phase of growth of derivatives of the wild-type (open symbols) and *mxiD* (closed symbols) strains carrying the *virA-lacZ* fusion. Samples were then collected at 5 min intervals and assayed for β-galactosidase activity. For both strains, no increase in β-galactosidase activity was detected in the absence of Congo red.

Figure 4 depicts transcription of the *virA-lacZ* fusion by intracellular bacteria. Intracellular bacteria recovered after various times of infection of HeLa cells by SF1001 (*virA-lacZ*) were numbered by plating (open symbols) and used to assay β-galactosidase activity (closed symbols).

Production of most bacterial virulence factors is tightly regulated in response to environmental signals. In *Shigella*, for example, both the temperature and the osmolarity of the growth medium modulate transcription of genes involved in entry of the bacteria into epithelial cells (Bernardini *et al.*, 1990, "The two-component regulatory system OmpR-EnvZ controls the virulence of *Shigella*.", "*J. Bacteriol.*, **172**:6274-6281 ; Maurelli *et al.*, 1984, "Temperature-dependent expression of virulence genes in *Shigella* species.", *Infect. Immun.*, **43**:195-201). In addition, contact of *Shigella* with epithelial cells (Ménard *et al.*, 1994; Watarai *et al.*, 1995) and exposure of the bacteria to Congo red (Sankaran *et al.*, 1989, "Congo-red mediated regulation of levels of *Shigella flexneri* 2a membrane proteins.", *Infect. Immun.*, **57**:2364-2371; Parsot *et al.*, 1995, "Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures." *Mol. Microbiol*, **16**, 291-300.; Bahrani *et al.*, 1997) or bile salts (Pope *et al.*, 1995) activate secretion of IpaB and IpaC.

The *Shigella* Model

Shigella was used as a model system for application of the methods of this invention. Members of the genus *Shigella* cause bacillary dysentery in humans by invading the colonic epithelial mucosa and inducing a strong inflammatory response (LaBrec *et al.*, 1964). *In vitro*, cell invasion involves two steps : entry and intercellular dissemination. Genes involved in both steps are carried on a 200-kb virulence plasmid (reviewed by Hale, 1991: Parsot, 1994). A 31-kb fragment of this plasmid is necessary and apparently sufficient for entry into epithelial cells (Maurelli *et al.*, 1985; Sasakawa *et al.*, 1988). This fragment is organized in two divergently transcribed regions which, schematically, encode secreted proteins, the IpaA-D proteins, and a type III secretion system, the Mxi-Spa secretion apparatus. The first region contains eight genes, including *ipaBCDA*, which are transcribed from a promoter located upstream from *icsB*. The second region contains 20 genes, designated *ipg, mxi*, and *spa*, which are clustered in large operons. Inactivation of *ipa*, *mxi*, and *spa* genes leads to a non-invasive phenotype, due to either loss of effector proteins (Sasakawa *et al.*, 1989; Ménard *et al.*, 1993) or failure to secrete them (Andrews and Maurelli, 1992; Venkatesan *et al.*, 1992; Allaoui *et al.*, 1993b; Sasakawa *et al.*, 1993).

Bacterial strains and growth media

All *Shigella flexneri* strains identified in Table I are derivatives of the wild-type strain M90T (Sansonetti *et al.*, 1982). Bacteria were grown in Luria-Bertani (LB) medium or tryptic soy (TCS) broth. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 30 μg/ml; and streptomycin, I00 μg/ml. Congo red (SERVA, Heidelberg, Germany) was used to induce secretion by bacteria growing in LB medium.

Constitutively secreting strains

Only a small proportion of IpaA-D proteins is secreted by wild-type *Shigella* growing in laboratory media. Inactivation of *ipaD* enhances secretion of IpaA, IpaB, IpaC, and about 15 other proteins (Ménard *et al.*, 1994; Parsot *et al.*, 1995). These latter proteins are absent or barely detectable in the medium of the wild-type strain unless Congo red, a dye that induces secretion (Bahrani *et al.*, 1997), is present in the culture medium (Parsot *et al.*, 1995).

Inactivation of either *ipaB* or *ipaD* and deletion of the *ipaB, C, D,* and *A* genes lead to the secretion of about 15 proteins that associate in the extracellular medium (Parsot *et al.*, 1995). Aggregates containing proteins secreted by the Δ*ipaBCDA* mutant (SF635) were used to immunize mice and the resulting anti-serum was tested by Western blotting on extracts of whole cultures, bacterial pellets, and culture supernatants of M90T (wild-type), SF622 (*ipaD*), SF635 (Δ*ipaBCDA*), SF634 (*ipaD mxiD*), and BS176 (a virulence-plasmid cured-stain). The serum reacted most strongly with a 46-kDa protein. This protein was present in high amounts in extracts of *ipaD* and Δ*ipaBCDA* strains; was present in low amounts in extracts of wild-type and *ipaD mxiD* strains; and was not present in extracts of the virulence plasmid-cured strain (Figure 1). SDS-PAGE analysis and Coomassie blue staining also revealed that a protein, or possibly a mixture of proteins of about 60 kDa was present in higher amounts in extracts of the *ipaD* and Δ*ipaBCDA* strains than in extracts of the wild-type and *ipaD mxiD* strains (Figure 1). These results suggested that production of 46-kDa and 60-kDa secreted proteins was increased in the constitutively secreting *ipaD* and Δ*ipaBCDA* strains as compared to the wild-type and secretion deficient *ipaD mxiD* strains.

Characterization of the gene encoding the 46-kDa secreted protein

The 46-kDa protein secreted by the Δ*ipaBCDA* mutant was transferred onto a PVDF membrane and subjected to Edman degradation and proteolysis by endolysin. The N-terminal sequence of the protein was identified as M-Q-T-S-N-I-T-N-H-E and those of two internal peptides as I-I-T-F-G-I-Y-S-P-H-E-T-L-A and V-H-T-I-T-A-P-V-S-G-N. Oligonucleotides based on the N-terminal sequence and one internal peptide were used to screen, by Southern blotting, a set of overlapping cosmids representing the entire virulence plasmid. Both probes hybridized to a 6.4 kb *Hind*III fragment of cosmid pCos3 which was then cloned into pUC19 to give rise to pBD3 (Figure 2).

Subcloning experiments and Southern blot analysis of recombinant plasmids using oligonucleotides as probes allowed us to localize the gene encoding the 46-kDa protein on a 3.2-kb *Hind*II-*Hind*III fragment located upstream from *icsA* (Bernardini *et al.*, 1989; Lett *et al.*, 1989). Sequence analysis revealed an open reading frame (ORF) starting 487 bp upstream from the *icsA* translation start codon and oriented in the opposite direction. Amino acid sequences deduced from positions 43 to 71, 159 to 200, and 442 to 473 of the ORF were identical to those determined for the N-terminal end and the two internal peptides of the secreted protein. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under the accession number AF047364. The deduced sequence of the 46-kDa protein was identical to that of VirA, a secreted protein encoded by the virulence plasmid of a *S. flexneri* strain of serotype 2a (Uchiya *et. al.*, 1995) and, therefore, the corresponding gene of *S. flexneri* 5 was designated *virA*. No other ORF was detected immediately upstream or downstream from *virA*. Restriction analysis of overlapping cosmids indicated that *virA* was located about 10 kb downstream from the *spa* operon (Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993) on the virulence plasmid pWR100.

Characterization of the gene encoding a 60-kDa secreted protein

The 60-kDa proteins which were secreted in high amount by the Δ*ipaBCDA* strain were transferred onto a PVDF membrane and the lower part of the band was used for N-terminal sequence determination and proteolysis by endolysin. Analysis of the N-terminal sequence indicated that the sample contained two proteins; the sequence of the major species was determined as M-L-P-I-N-N-N-F-S-L-P-Q. The sequence of an internal peptide was determined as Y-E-M-L-E-N-E-Y-P-Q-R-V-A-D-R, which was almost identical to a fragment of the constant region of members of the IpaH family. IpaH proteins are characterized by a constant C-terminal region of about 300 residues which is preceded by a variable N-terminal region composed of repetitive motifs (Hartman *et al.*, 1990; Venkatesan *et al.*, 1991). The N-terminal sequence of the 60-kDa secreted protein was different from those deduced from the 5' end of *ipaH7.8, ipaH4.5, ipaH2.5* and *ipaH1.4* (Hartman *et al.*, 1990; Venkatesan *et al.*, 1991), which suggested that this protein might correspond to the fifth IpaH protein, IpaH9.8, whose gene had not been sequenced yet.

Southern blot analysis using a probe derived from the constant region of *ipaH* genes indicated that *ipaH9.8* was present in cosmid pCos87. Deletion derivatives of pCos87 were constructed to give rise to pBD4 (Figure 2), whose 2.4-kb insert was entirely sequenced. The amino acid sequences deduced from positions 40 to 75 and 1477 to 1521 of the ORF identified by sequence analysis were identical to those of the N-terminal end and of the internal peptide of the 60-kDa secreted protein. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under the accession number AFO47365. The *ipaH9.8* gene encodes a 545-residue protein with a deduced Mr of 61,886. No ORFs were identified upstream or downstream from *ipaH9.8*. Restriction analysis of overlapping cosmids indicated than *ipaH9.8* was located 45 kb downstream from the *spa* operon.

DNA analysis, PCR, plasmid construction, and transformation of *E.coli* and *S. flexneri* strains were performed according to standard methods. Nucleotide sequences were determined by the dideoxy chain termination procedure on alkaline-denaturated plasmid DNA. Overlapping cosmids representing the entire virulence

plasmid were previously constructed by inserting 40-kb fragments of pWR100 into the vector pJB8 (Maurelli *et al.*, 1985).

<u>Inactivation of *ipaD* increases transcription of *virA* and *ipaH* genes</u>

Western blot analysis indicated that a higher amount of VirA was produced by the *ipaD* mutant than by the wild-type strain (Figure 1). To investigate *virA* transcription, we constructed a *virA-lacZ* transcriptional fusion. Plasmid pLAC4 (Figure 2) was constructed by cloning a 1.5-kb *Xba*I-*Eco*RI fragment that contains the 5' part of *icsA*, the *icsA-virA* intergenic region, and the 5' part of *virA*, into the *Sma*I site located upstream from the *lacZ* reporter gene in the suicide plasmid pLAC1 that confers resistance to ampicillin (Allaoui *et al.*, 1992). (Plasmid pLAC4 was deposited in the Collection Nationale de Cultures de Microorganismes in Paris, France on May 13, 1998 under accession No. I-2105.) pLAC4 was then transferred by conjugation into M90T-Sm and SF622 (*ipaD*2) to produce recombinant strains SF1001 and SF1002. Since pLAC4 does not replicate in *S. flexneri*, the Ap^r clones arose through homologous recombination between the identical sequences carried by the virulence plasmids M90T-Sm or SF622 and the recombinant p1asmid pLAC4, thereby placing the *lacZ* reporter gene under the control of the *virA* promoter. Expression of the *virA-lacZ* fusion was 17 times higher in the *ipaD*$^-$ strain as compared to the *ipaD*$^+$ strain (Table II), indicating that the increased production of VirA by the *ipaD* mutant was due to an increased transcription of *virA*. Southern analysis confirmed the structure of the pWR100 derivatives carrying the *virA-lacZ* transcriptional fusion in recombinant strains designated SF1001 (*virA-lacZ virA*$^+$*ipaD*$^+$) and SF1002 (*virA-lacZ virA*$^+$*IpaD*$^-$).

Plasmid pLAC5 (Figure 2) was constructed by deleting a NdeI-EcoRI fragment from pLAC4 and thus contains a 380-bp fragment internal to the *virA* gene. To determine whether VirA is involved in the regulation of the *virA* promoter, pLAC5 was integrated at the *virA* locus of the wild-type and *ipaD* to produce recombinant strains SF1003 and SF1004. Integration of pLAC5 into the *virA* locus of pWR100 also placed the *lacZ* gene under the control of the *virA* promoter but, unlike that of pLAC4,

led to the disruption of the *virA* gene. Inactivation of *virA* had no effect on transcription of the *virA-lacZ* fusion in either the *ipaD*$^+$ or *ipaD*$^-$ backgrounds, indicating that *virA* was not autoregulated. Southern analysis confirmed the structure of the pWR100 derivatives carrying the *virA-lacZ* transcriptional fusion in recombinant strains designated SF1003 (*virA-lacZ virA*$^-$*IpaD*$^+$) and SF1004 (*virA-lacZ virA*$^-$*ipaD*$^-$).

To analyze transcription of the various *ipaH* genes, the constant region of *ipaH9.8* was amplified using the polymerized chain reaction (PCR) technique, and the PCR product was cloned between the *Kpn*I and *Xba*I sites that are located upstream from the *lacZ* gene in the suicide vector pLAC2 (Allaoui *et al.*, I 993a) to construct plasmid pLAC6. pLAC6 was then transferred by conjugation into M90T-Sm (wild-type) and SF622 (*ipaD*). Since pLAC6 carried the constant region of *ipaH*, integration of the suicide plasmid could occur into any of the five *ipaH* genes carried on the virulence plasmid. In each case, the *lacZ* reporter gene is placed under the control of the promoter of the *ipaH* gene into which the plasmid is integrated. Transconjugants were screened by Southern blot analysis of their virulence plasmid digested by *Hind*III using a probe from the *ipaH* constant region. The strains were designated SF1005 (*ipaH9.8-lacZ ipaD*$^+$), SF1006 (*ipaH9.8-lacZ ipaD*$^-$), SF1007 (*ipaH7.8-lacZ ipaD*$^+$), SF1008 (*ipaH4.5-lacZ ipaD*$^+$), SF1009 (*ipaH4.5-lacZ ipaD*$^-$), SF 1010 (*ipaH1.4-lacZ ipaD*$^+$), SF1011 (*ipaH1.4-lacZ ipaD*$^-$). The *ipaH2.5-lacZ* fusion in the wild-type background as well as the *ipaH7.8-lacZ* and *ipaH2.5-lacZ* fusions in the *ipaD* background were not obtained. Expression of *ipaH9.8-*, *ipaH4.5-*, and *ipaH1.4-lacZ* fusions was low in derivatives of the wild-type strain and was increased 5-20 times in derivatives of the *ipaD* mutant (Table II).

To investigate transcription of genes of the entry region, we used *lacZ* transcriptional fusions in *icsB* and *ipaA*, which are the first and last genes of the *ipaBCDA* operon, respectively, and in *ipgD* and *mxiD*, which are the first and 12th genes of the *mxi* operon, respectively (Figure 2). These fusions were constructed in both the wild-type and *ipaD* strains. For example, construction of the *ipgD-lacZ* fusion was achieved using the suicide plasmid pLAC3 (Allaoui *et al.* 1993a). The

recombinant plasmid pLAC3 (Allaoui et al., 1993) was constructed by cloning a 1.4-kb *SspI* fragment that contains the 5' part of *icsB*, the *icsB-ipgD* intergenic region, and the 5' part of *ipgD* (Allaoui et al., 1992, 1993) into the *SmaI* site located upstream from the *lacZ* reporter gene in the vector pLAC1 (Allaoui et al., 1992). (Plasmid pLAC3 was deposited in the Collection Nationale de Cultures de Microorganismes in Paris, France on May 13, 1998 under accession No. I-2104.) pLAC3 was then transferred by conjugation into M90T-Sm and SF622 (*ipaD2*) to produce recombinant strains SF134 and SF806. Since pLAC3 does not replicate in *S. flexneri*, the Ap^r clones arose through homologous recombination between the identical sequences carried by the virulence plasmids M90T-Sm or SF622 and the recombinant p1asmid pLAC3, thereby placing the *lacZ* reporter gene under the control of the *virA* promoter. Southern analysis confirmed the structure of the virulence plasmid carrying the *ipgD-lacZ* transcriptional fusion in the recombinant strains designated SF134 (*ipgD-lacZ ipaD*$^+$) and SF806 (*ipgD-lacZ ipaD*$^-$). Integration of the suicide plasmids used to construct these fusions did not affect the secretion phenotype of recombinant strains. For each fusion, similar amounts of β-galactosidase were present in derivatives of the wild-type and *ipaD* strains, indicating that transcription of these genes was not affected by inactivation of *ipaD* (Table II).

<u>Congo red increases transcription of *virA* and *ipaH* genes</u>

Secretion of IpaB and IpaC is enhanced when bacteria grow in the presence of Congo red (Parsot *et al.*, 1995). To investigate the effect of Congo red on *virA* transcription, we assayed the β-galactosidase activity in strain SF1001 (*virA-lacZ ipaD*$^+$) after growth in the presence of various concentrations of Congo red. Transcription of the *virA-lacZ* fusion was low at concentrations of dye up to 20 μg/ml and then increased with the concentration of the dye to reach a plateau at about l00 μg/ml of Congo red. Likewise, about 3-12 times more β-galactosidase activity was present in strains carrying *ipaH9.8-*, *ipaH7.8-*, *ipaH4.5-*, and *ipaH1.4-lacZ* fusions after growth in the presence of 100 μg/ml of Congo red (Table II). In contrast, transcription of *icsB-*, *ipaA-*, *ipgD-*, and *mxiD-lacZ* fusions was not affected by the presence of Congo red in the growth medium (Table II).

<u>Secretion is required for activation of *virA* transcription</u>

To determine whether regulation of *virA* transcription was dependent on the type III secretion machinery, we compared the β-galactosidase activities produced by the *virA-lacZ* fusion in derivatives of wild-type (SF1001) and *mxiD* (SF1012) strains during growth in the presence of Congo red and we compared the production of VirA in *ipaD* and *mxiD ipaD* strains.

The presence of Congo red in the growth medium of the derivative of the *mxiD* strain carrying the *virA-lacZ* fusion did not lead to an increase in β-galactosidase activity (Table II), and lesser amounts of VirA were present in the *ipaD mxiD* strain as compared to the *ipaD* strain (Figure I). This indicated that activation of the *virA* promoter in response to Congo red and inactivation of *ipaD* required the integrity of the secretion machinery.

To investigate kinetics of activation of the *virA* promoter, Congo red (100μg/ml) was added to the growth medium during the exponential phase of growth of derivatives of the wild-type and *mxiD* strains carrying the *virA-lacZ* fusion. Samples were then collected at 5 min intervals and assayed for β-galactosidase activity. An increase in the β-galactosidase specific activity was detected 10 min after addition of the dye to the medium of the derivative of the wild-type strain, whereas no transcriptional activation of the *virA-lacZ* fusion was detected in the derivative of the *mxiD* mutant (Figure 3).

These results differentiated the *virA* and *ipaH* genes, the transcription of which was increased after growth in the presence of Congo red or by inactivation of *ipaD*, from the genes of the entry region, the transcription of which was apparently constitutive with respect to these parameters. Moreover, this suggested that transcription of the *virA* and *ipaH* genes was regulated by the Mxi-Spa secretion machinery, since (i) conditions leading to an enhanced transcription of these genes were the same as those known to increase secretion through the Mxi-Spa secretion machinery, and (ii) in these conditions, the secretion machinery was required for the

enhanced transcription of the *virA-lacZ* fusion and for the enhanced production of the VirA protein.

Transcription of *virA*- and *ipaH-lacZ* fusions upon entry and during intracellular multiplication

To investigate *virA* and *ipaH* transcription during infection of epithelial cells, we measured the β-galactosidase activity that was present in bacteria shortly after entry into epithelial cells. HeLa cells were infected as previously described (Sansonetti *et al.*, 1986). Briefly, cells were infected for 30 minutes to allow entry and then treated with gentamicin for 30 minutes to kill extracellular bacteria. Infected cells were then washed to remove killed bacteria and lyzed, and intracellular bacteria were recovered by centrifugation. The number of intracellular bacteria was determined by plating and the β-galactosidase activity present in these bacteria was assayed by using MUG as a substrate. The specific activity was first expressed in units of fluorescence per bacterium and then converted into Miller units. For the strain carrying the *ipgD-lacZ* fusion, chosen as a representative of genes which were expressed constitutively *in vitro*, the β-galactosidase activity present within intracellular bacteria recovered after 60 min of infection was similar to that found after growth in laboratory medium (Table III). This confirmed that, following gentamicin treatment, washes of infected cells were sufficient to remove killed extracellular bacteria which, otherwise, could have contributed to the total β-galactosidase activity without being numbered by plating. For strains carrying the *virA*- and *ipaH-lacZ* fusions, the β-galactosidase activity was 6 to 30 times higher in intracellular bacteria than in bacteria grown *in vitro* (Table III). This indicated that transcription of *virA, ipaH9.8, ipaH7.8*, *ipaH4.5* and *ipaH1.4* had been induced upon entry or shortly thereafter.

To investigate *virA* transcription during growth in the intracellular compartment, infected cells were lyzed after various periods of incubation in the presence of gentamicin and intracellular bacteria were counted by plating and assayed for β-galactosidase activity. The number of intracellular bacteria carrying the *virA-lacZ* fusion increased with the time of incubation, which was consistent with their intracellular multiplication (Figure 3). In contrast, the specific β-galactosidase activity

present in these bacteria decreased steadily, suggesting that the decrease in specific activity was due to bacterial multiplication. Similarly, the β-galactosidase activity present in bacteria carrying the various *ipaH-lacZ* fusions was 6 to 13 times lower after 150 min of infection as compared to the activity present after 60 min of infection (Table III). These results suggested that the *virA*- and *ipaH-lacZ* fusions had not been transcribed between 60 and l50 min of infection. In contrast, for the strain carrying the *ipgD-lacZ* fusion, similar amounts of β-galactosidase were present after 60 and 150 min of infection (Table III), suggesting that the intracellular compartment had no effect on *ipgD* transcription.

<u>Protein analysis</u>

All protein analyses were carried out according to the following protocol. Aggregated proteins were collected from the culture medium of SF635 (*Δipa*) and solubilized in 0.1% SDS. Mice were injected twice with this preparation, at one week interval. Sera were collected the fourth week, pooled, and absorbed on sonicated extracts of BS176.

Bacteria in the exponential phase of growth were harvested by centrifugation at 14,000 g for 10 min. Crude extracts were obtained from the bacterial pellet, and proteins present in the culture supernatant were precipitated by the addition of 1/10 (vol/vol) trichloracetic acid. Electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described (Laemmli, 1970). After electrophoresis, proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane.

Immunoblotting procedures were carried out with mouse polyclonal anti-filaments antibodies. Horseradish peroxidase-labelled goat anti-mouse antibodies were used as secondary antibodies and visualized by enhanced chemiluminescence. The N-terminal sequence of VirA and IpaH9.8 and that of internal peptides, which were obtained by endolysin digestion and purified by chromatography, were determined by the Edman degradation procedure.

The β-galactosidase activity present in bacteria growing in laboratory media was assayed by using the substrate *o*-nitro-phenyl-β-D-galactoside (ONPG) as described (Platt *et al.*, 1972). The β-galactosidase activity present in intracellular bacteria was assayed by using the substrate 4-methyl-umbelliferyl-β-D-galactoside (MUG) as described (Klarsfeld *et al.*, 1994). Fluorescence was measured by using a Dynatec apparatus, with 365 nm excitation and 450 nm emission wavelengths. Activities were computed as fluorescence units per hour per bacterium; four fluorescence units were equivalent to one Miller unit and all results are presented in Miller units.

Genes under transcriptional control of type III secretion machinery

Using *lacZ* transcriptional fusions, we have investigated transcription of *virA*, of four members of the *ipaH* family, and of the *ipaBCDA* and *mxi* operons. We present evidence that transcription of *virA* and of four *ipaH* genes, but not that of the *ipaBCDA* and *mxi* operons, is increased when secretion through the type III secretion machinery is enhanced in response to addition of Congo red to the growth medium and to inactivation of *ipaD*. In addition, transcription of *virA*- and *ipaH-lacZ* fusions was activated during entry of bacteria into epithelial cells.

We used *lacZ* fusions to investigate transcription of *virA*, *ipaH9.8*, *ipaH7.8*, *ipaH4.5*, and *ipaHl.4*, as well as that of operons located in the entry region. Transcription of genes of the entry region was high in derivatives of the wild-type strain and was not increased in derivatives of the *ipaD* mutant or after growth in the presence of Congo red. These results indicate that: (i) the increased secretion observed with the wild-type strain growing in the presence of Congo red and with the *ipaD* mutant is not due to an increased transcription of the *mxi* operon; and (ii) transcription of *mxi* and *ipaBCDA* operations is the same whether the secretion machinery is poorly active (in the wild-type strain), or deregulated (by addition of Congo red or inactivation of *ipaD*). This transcriptional analysis and previous Western blot analysis, which indicated that similar amounts of IpaB and IpaC were present in wild-type, *ipaD*, and *mxiD* strains (Allaoui *et al.*, 1993b; Ménard *et al.*,

1993), suggest that expression of genes of the entry region is not controlled by the secretion machinery. In contrast, transcription of the *virA* and *ipaH* genes was low in derivatives of the wild-type strain and was increased during growth in the presence of Congo red and in derivatives of the *ipaD* mutant. These results, together with the low production of VirA in the *ipaD mxiD* mutant and the low transcription of *virA* in the *mxiD* mutant growing in the presence of Congo red, indicate that the secretion machinery is involved in the control mechanism of the *virA* promoter and suggest that transcription of *virA* and of four copies of the *ipaH* family is enhanced in response to an active secretion through the type III apparatus.

Similar amounts of β-galactosidase were present in bacteria carrying the *ipgD-lacZ* fusion prior to and after 60 min of infection. In contrast, the amount of β-galactosidase present in bacteria carrying *virA* and *ipaH-lacZ* fusions was about 10 times higher after 60 min of infection than prior to infection. Due to the period of incubation in the presence of gentamicin which is required to eliminate extracellular bacteria, we could not investigate whether *virA* and *ipaH* transcription was activated upon contact with or shortly after entry into epithelial cells. Only low amounts of β-galactosidase were present in bacteria carrying *virA*- and *ipaH-lacZ* fusions after 150 min of infection, which suggests that the *virA* and the *ipaH* genes had not been transcribed between 60 and 150 min of infection. Since there is a correlation between *virA* and *ipaH* transcription and active secretion, these results suggest that secretion might not be active when bacteria are multiplying in the cytoplasm of HeLa cells. Alternatively, signals other than secretion might affect negatively transcription of the *virA* and *ipaH* genes in the intracellular compartment.

The mechanism involved in the transcriptional control of the *virA* and *ipaH* genes in response to active secretion is not known yet. The low transcription of *virA* by the *virA* mutant indicates that *virA* is not autoregulated and the low production of VirA by the *ipaD mxiD* mutant suggests that IpaD is not the effector of the regulation of the *virA* promoter. When the secretion apparatus is inactive, a negative regulator might accumulate in the cytoplasm and repress *virA* and *ipaH* transcription. Secretion of this regulator, due to the lack of IpaD or in response to external inducers,

would decrease its cytoplasmic concentration, thereby leading to the transcriptional activation of its target promoters. Secretion of a negative regulator as a mechanism for the control of gene expression has been documented in *Salmonella* and *Yersinia*. In *S. typhimurium*, transcription of the flagellin gene by an RNA polymerase containing the alternate sigma factor δ^{28} requires the integrity of the basal-hook body complex which constitutes an export apparatus related to type III secretion machineries. Secretion of the anti-sigma factor FlgM allows transcription of the flagellin gene by a δ^{28}-containing RNA polymerase, thus coupling flagellin expression to flagellar assembly (Hughes *et al.* 1993; Kutsukake *et al.*, 1994). In *Yersinia*, expression of the *yop* genes is down regulated when Yop secretion is compromised (Cornelis *et al.*, 1987) and secretion of LcrQ via the type III secretion apparatus has been proposed to lead to the transcriptional activation of *yop* promoters by a mechanism which has not been characterized yet (Pettersson *et al.*, 1996). Differences in the transcriptional regulation of genes encoding proteins secreted by the type III secretion machinery of *Shigella* are likely to reflect differences in the functional role of these secreted proteins during infection.

Table I. *Shigella* strains

Strain	Genotype	Reference
M90T	wild type	Sansonetti *et al.*, 1985
M90T-Sm	spontaneous Sm^R derivative of the M90T	Allaoui *et al.*, 1992
BS176	plasmidless derivative of M90T	Sansonetti *et al.* 1985
SF132	*icsB-lacZ* in M90T-Sm	Allaoui *et al.*, 1992
SF134	*ipgD-lacZ* in M90T-Sm	Allaoui *et al.*, 1993a (C.N.C.M. No. I-2016)*
SF401	*mxiD*	Allaoui *et al.*, 1993b
SF403	*mxiD-lacZ* in M90T-Sm	Allaoui *et al.*, 1993b
SF622	*ipaD*	Ménard *et al.*, !993
SF623	*ipaA-lacZ* in M90T-Sm	Ménard *et al.*, 1993
SF624	*ipaA-lacZ* in SF622 (*ipaD*)	Ménard *et al.*, 1993
SF634	*ipaD mxiD*	Ménard *et al.*, 1994
SF635	*ΔipaBCDA*	Parsot *et al.*, 1995
SF803	*icsB-lacZ* in SF622 (*ipaD*)	
SF806	*ipgD-lacZ* in SF622 (*ipaD*)	(C.N.C.M. No. I-2017)*
SF808	*mxiD-lacZ* in SF622 (*ipaD*)	
SF1001	*virA-lacZ* in M90T-Sm (*virA*$^+$)	(C.N.C.M. No. I-2018)*
SF1002	*virA-lacZ* in SF622 (*virA*$^+$)	(C.N.C.M. No. I-2019)*
SF1003	*virA-lacZ* in M90T-Sm (*virA*$^-$)	
SF1004	*virA-lacZ* in SF622 (*virA*$^-$)	
SF1005	*ipaH9.8-lacZ* in M90T-Sm	
SF1006	*ipaH9.8-lacZ* in SF622 (*ipaD*)	
SF1007	*ipaH7.8-lacZ* in M90T-Sm	
SF1008	*ipaH4.5-lacZ* in M90T-Sm	
SF1009	*ipaH4.5-lacZ* in SF622 (*ipaD*)	
SF1010	*ipaH1.4-lacZ* in M90T-Sm	
SP1011	*ipaH1.4-lacZ* in SF622 (*ipaD*)	
SF1012	*virA-lacZ* in SF401 (*mxiD*)	

* Deposited in the Collection Nationale de Cultures de Microorganismes in Paris, France on May 13, 1998.

Table II. Expression of *lacZ* transcriptional fusions by bacteria growing *in vitro*

β-galactosidase activity (Miller units)[a]

Fusion	*ipaD*⁺	*ipaD*⁻	Ratio I[b]	*ipaD*⁺ + CR	Ratio II[c]
virA-lacZ	16	280	17	280	17
virA-lacZ mxiD	17	NA	NA	18	1.1
ipaH9.8-lacZ	28	580	21	325	12
ipaH7.8-lacZ	20	NA	NA	235	12
ipaH4.5-lacZ	31	360	12	110	3.5
ipaH1.4-lacZ	53	280	5.3	270	5.1
ipaA-lacZ	485	510	1.1	390	0.8
icsB-lacZ	290	305	1.1	285	1.0
mxiD-lacZ	275	260	0.9	320	1.2
ipgD-lacZ	450	400	0.9	475	1.1

[a]Activities are the means of at least three independent experiments. Standard deviations are within 25% of the reported values.

[b]Activity present in *ipaD*⁻ strains versus activity present in *ipaD*⁺ strains.

[c]Activity present in derivatives of the *ipaD*⁺ strain grown in the presence of Congo red versus activity present in the same strains grown in the absence of Congo red.

NA not applicable.

Table III. Expression of *lacZ* transcriptional fusions by intracellular bacteria

β-galactosidase activity (Miller units)[a]

Fusion	*in vitro*	60min of infection	Ratio I[b]	150min of infection	Ratio II[c]
ipgD-lacZ	450	490	1.1	463	1.1
virA-lacZ	16	280	18	49	5.7
ipaH9.8-lacZ	28	350	13	49	7.1
ipaH7.8-lacZ	20	590	30	150	3.9
ipaH4.5-lacZ	31	280	9.0	21	13.3
ipaH1.4-lacZ	53	300	5.7	49	6.1

[a]Activities are the means of at least three independent experiments. Standard deviations are within 25% of the reported values.

[b]Activity present after 60 min of infection versus activity present in bacteria grown *in vitro*.

[c]Activity present after 60 min of infection versus activity present after 150 min of infection

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We claim:

1. A method for screening molecules that activate or inhibit type III secretion machinery in gram-negative bacteria comprising the steps of :

a) exposing gram-negative bacterial cells to a sample molecule, wherein said bacterial cells contain a reporter gene transcriptionally fused to a promoter of a gene activated or regulated by the type III secretion machinery; and

b) detecting the presence or activity of the product of the reporter gene.

2. The method according to claim 1, wherein the gram-negative bacteria is selected from the group consisting of *Shigella*, *Salmonella*, *Yersinia*, *Escherichia*, *Pseudomonas*, *Xanthomonas*, *Ralstonia*, and *Erwinia*.

3. The method according to claim 1, wherein the gram-negative bacteria is *Shigella*.

4. The method according to any one of claims 1-3, wherein the reporter gene is selected from the group consisting of *lacZ*, *phoA*, *gfp*, and *luxAB*.

5. The method according to claim 4, wherein the reporter gene is *lacZ* and the product of the reporter gene is β-galactosidase.

6. The method according to any one of claims 1-3, wherein the promoter is from a gene selected from the group consisting of *virA*, *ipaH*, *ipgD*, *icsB*, *ipaA*, and *mxiD*.

7. The method according to claim 6, wherein the promoter is from a gene selected from the group consisting of *ipaH9.8*, *ipaH7.8*, *ipaH4.5*, and *ipaH1.4*.

8. The method according to claim 6, wherein the promoter is from the *ipaH9.8* gene.

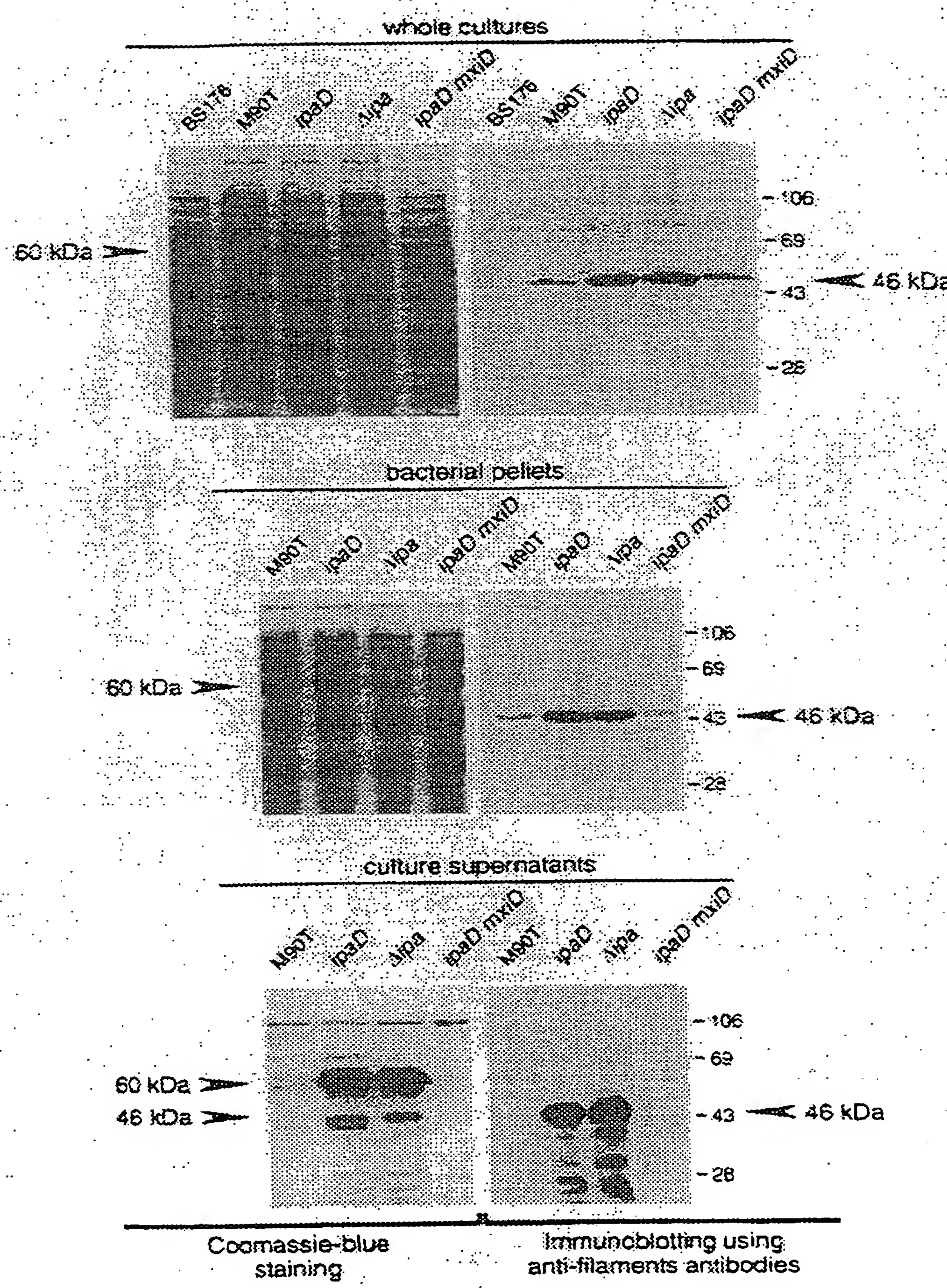
9. The method according to claim 6, wherein the promoter is from the *virA* gene.

10. The method according to claim 6, wherein the promoter is from the *ipgD* gene.

11. *Shigella* mutant strain SF134 (C.N.C.M. NO. I-2014).

12. *Shigella* mutant strain SF806 (C.N.C.M. No. I-2015).

13. *Shigella* mutant strain SF1001 (C.N.C.M. No. I-2018).
14. *Shigella* mutant strain SF1002 (C.N.C.M. No. I-2019).
15. Recombinant plasmid pLAC3 (C.N.C.M. No. I-2014).
16. Recombinant plasmid pLAC4 (C.N.C.M. No. I-2015).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ipaD
Δipa
ipaD mxiD
60 kDa
106
69
43
28
46 kDa
bacterial pellets
M90T
ipaD
Δipa
ipaD mxiD
M90T
ipaD
Δipa
ipaD mxiD
60 kDa
106
69
43
28
46 kDa
culture supernatants
M90T
ipaD
Δipa
ipaD mxiD
M90T
ipaD
Δipa
ipaD mxiD
60 kDa
46 kDa
106
69
43
28
46 kDa
Coomassie-blue staining
Immunoblotting using anti-filaments antibodies

FIG. 1

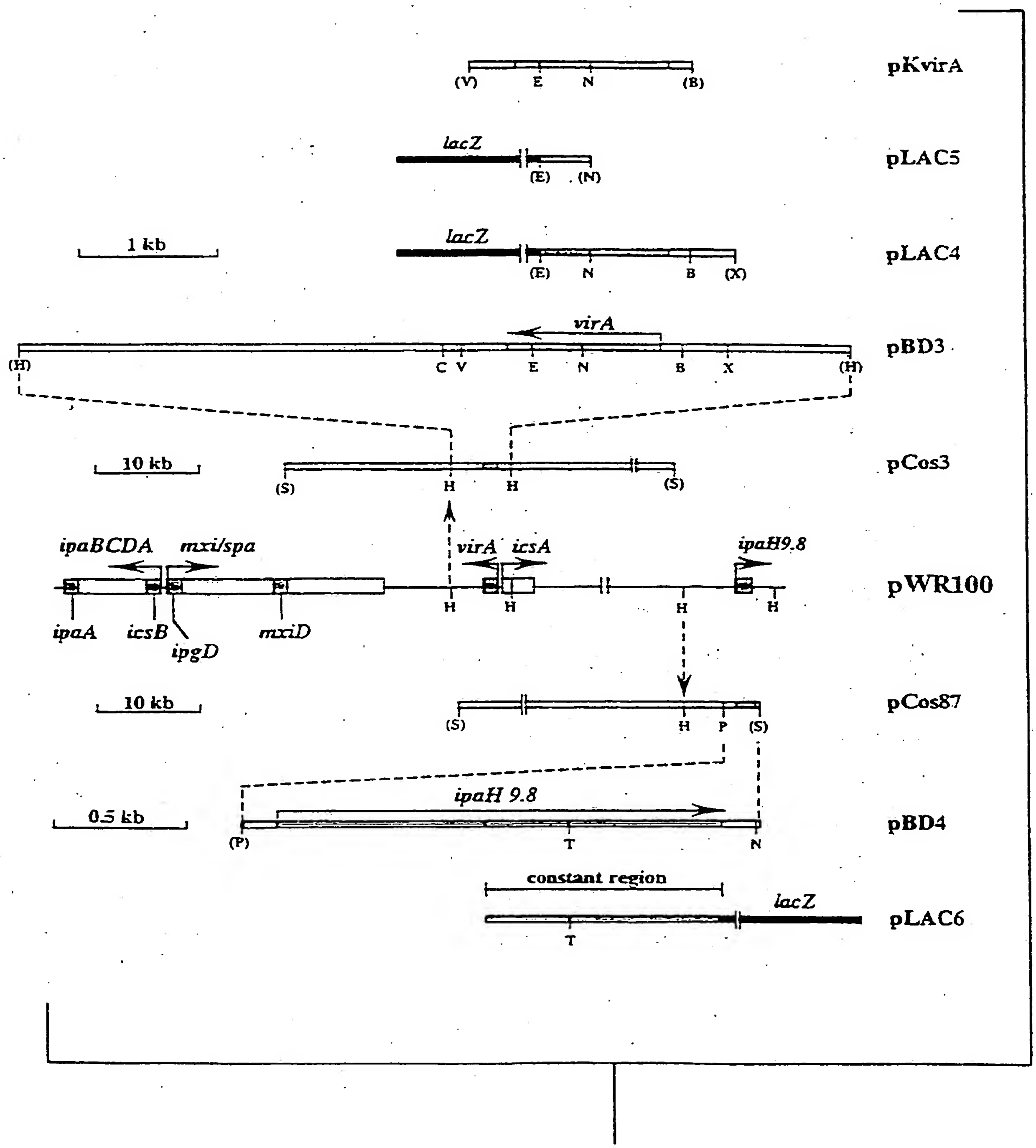
pKvirA
(V)
E
N
(B)
lacZ
pLAC5
(E)
(N)
1 kb
lacZ
pLAC4
(E)
N
B
(X)
virA
pBD3
(H)
C
V
E
N
B
X
(H)
10 kb
pCos3
(S)
H
H
(S)
ipaBCDA
mxi/spa
virA
icsA
ipaH9.8
pWR100
H
H
H
H
ipaA
icsB
ipgD
mxiD
10 kb
pCos87
(S)
H
P
(S)
0.5 kb
ipaH 9.8
pBD4
(P)
T
N
constant region
lacZ
pLAC6
T

FIG.2

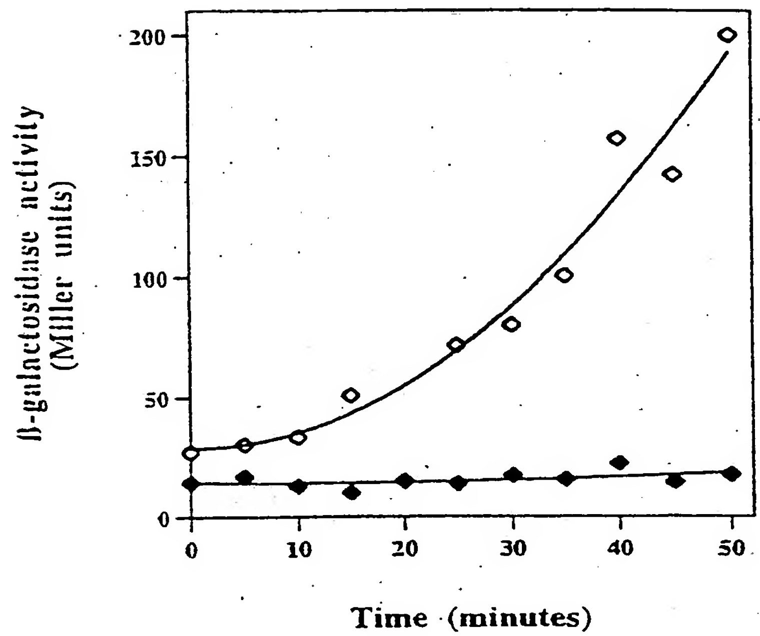
ß-galactosidase activity
(Miller units)
200
150
100
50
0
0
10
20
30
40
50
Time (minutes)

FIG.3

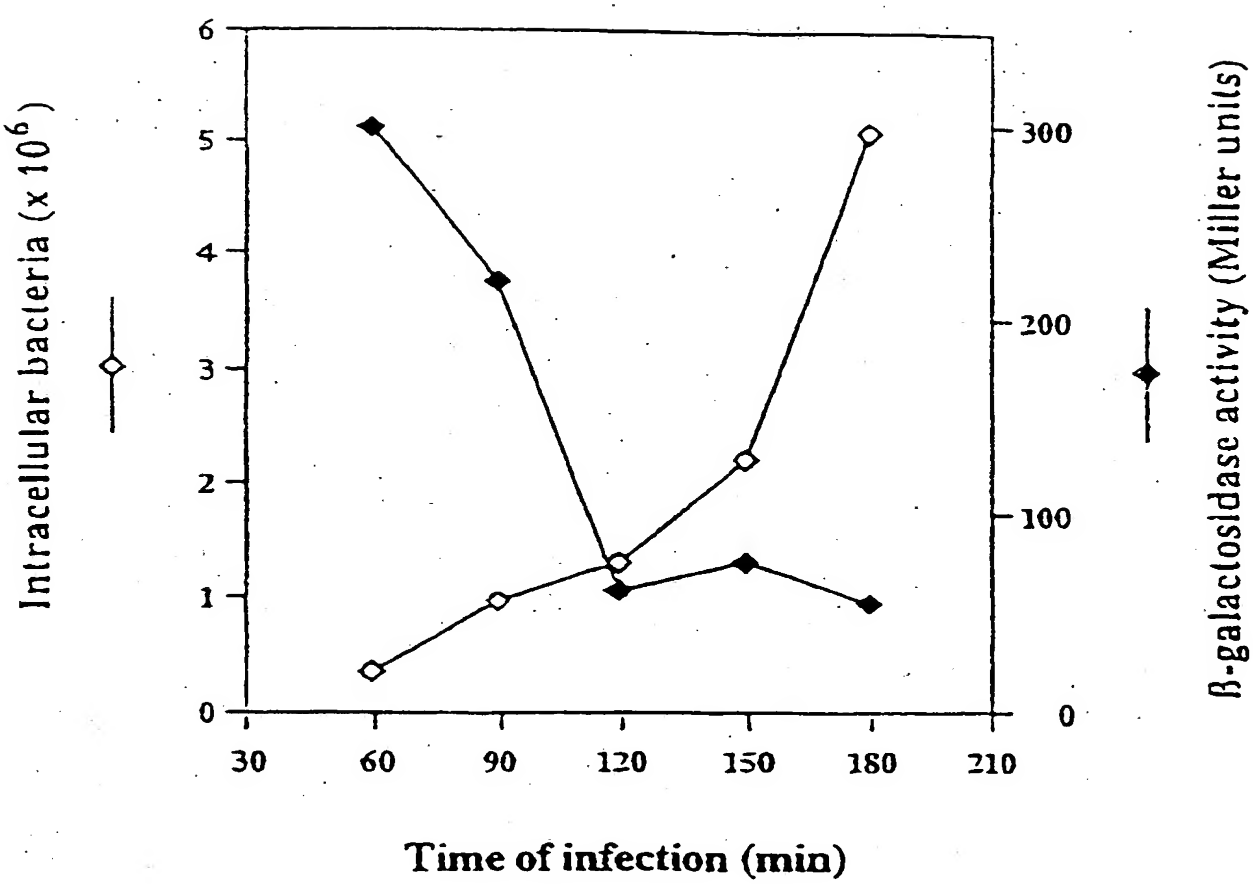
Intracellular bacteria (x 10^6)
β-galactosidase activity (Miller units)
Time of infection (min)
0
1
2
3
4
5
6
0
100
200
300
30
60
90
120
150
180
210

Fig. 4